May 1942

ET-192

United States Department of Agriculture
Bureau of Entomology and Plant Quarantine

METHOD FOR THE PREPARATION OF SPORE-DUST MIXTURES OF TYPE A MILKY DISEASE OF JAPANESE BEETLE LARVAE FOR FIELD INOCULATION

By S. R. Dutky,
Division of Fruit Insect Investigations

Studies at the Japanese Beetle Laboratory of the Bureau of Entomology and Plant Quarantine, at Moorestown, N. J., have indicated that the type A milky disease of Japanese beetle larvae (Popillia japonica Newm.) may be utilized for reduction of the larval population of this insect under field conditions. This disease is caused by the spore-forming bacterium Bacillus popilliae Dutky. The procedure described herein outlines the method which has been developed and is now in use for producing the spores in large quantities and preparing them in a suitable form for storage and field distribution. 1/ This summary outline has already been furnished to certain State agencies to aid them in disease-distribution projects which they were undertaking. It is now being included in the ET series to make it more generally available to those desiring information on the procedure followed in preparing the spores of the milky disease for distribution.

(1) STOCK CULTURES OF THE ORGANISMS

It has been found that films of dried blood from diseased larvae on glass microscope slides are satisfactory as stock cultures of these spores. Tests have shown these films to be suitable for use after storage for periods as long as 4 years. In a number of instances pure stock cultures of known identity and tested virulence have been furnished by the Bureau of Entomology and Plant Quarantine to official agencies interested in work with the milky disease of the Japanese beetle.

1/ Paten~~ts pending~~ 2,258,319

(2) PREPARATION OF THE INOCULUM

The first step is the preparation of a spore suspension to be used as the inoculum. This is done by removing the spores from the stock slides by moistening the dried blood films with sterile distilled water with the aid of a sterile pipette. This is facilitated by stroking the moistened film with the side of the pipette so as to bring the spore material into suspension. Then by holding the tip of the pipette against one corner of the slide and tilting the slide toward the pipette tip, the suspension is run into the inclined pipette. The suspension in the pipette is then run into a sterile test tube, and fresh distilled water is flooded over the slide. By the same procedure the second portion of water is removed from the slide and run into the test tube. Spore counts on the suspension in the test tube are made with a counting chamber, and the number of spores is adjusted to approximately 300 million per cc. The details of a procedure for making this adjustment easily and rapidly are given in the appendix (p. 6) following the main body of this paper.

About one-half cc. of this adjusted suspension is drawn into the pipette, and the pipette is placed in a horizontal position on a wire basket. The needle of the hypodermic syringe 2/ is inserted into the tip of the pipette, and the suspension is drawn into the syringe by pulling back the plunger. The syringe is then held vertically with the needle up, and the plunger is further withdrawn, admitting air into the syringe. While still in a vertical position the plunger is pushed forward, driving the entrapped air upward and out of the needle. The needle is then reintroduced into the tip of the pipette, and the suspension is discharged into the pipette until the plunger rests at the 0.35=cc. mark on the syringe. Extreme caution must be exercised to insure that no bubbles of air remain in the syringe, since even the smallest bubble will result in a failure of these small volumes to be injected into the grub because of the back pressure and compressibility of the air locked in the syringe. The syringe is then slipped into place in the injection block and the spring clip is fastened. The microinjector with the loaded syringe in place is shown in figure 1. The micrometer screw is turned forward until a droplet is forced from the end of the needle. This droplet is removed with a piece of absorbent cotton. An inoculating dosage is discharged into a dry, sterile test tube by depressing the pressure bar of the ratchet

---

2/ The syringe is part of the special "microinjector" developed at the Japanese Beetle Laboratory by Dutky and Fest for injecting larvae, and may be readily removed from the injector for loading.

mechanism. A 1-cc. portion of sterile distilled water is then pipetted into the test tube, washing the droplet of spore suspension from the side of the test tube and suspending the spores in this volume. Counts are made on this sample to check on the spore dosage.

(3) INJECTION OF LARVAE

In this work it is preferable to use full-grown larvae, although small third-instar and even second-instar larvae may be used if larger larvae are not available.

Larvae are injected in the following manner: The grub is held firmly but lightly between thumb and forefinger, the dorsal posterior portion outward, and guided toward the needle point. The grub is forced onto the needle point so that the needle enters in the dorsal portion of the suture between the second and third posterior abdominal segments. Care must be taken that the needle enters horizontally so as not to puncture the intestine. Care must also be taken to insure that as far as possible the site of puncture be free from adhering soil particles. The larva is then allowed to hang suspended on the needle during the injection. The injection is made at this time by depressing the pressure bar of the ratchet mechanism of the microinjector, which forces an inoculating dosage into the body cavity of the grub. This operation is illustrated in figure 2. By volume, the dosage approximates one three-hundredth of a cc. Since the inoculum is adjusted to 300 million spores per cc., the resulting spore dosage approximates 1 million spores.

(4) INCUBATION OF INJECTED LARVAE

Incubating boxes having a capacity of 500 larvae are used to hold the injected larvae during the period of incubation. These are equipped with metal cross-section separators which divide each of 5 layers into 100 compartments. After injection, the inoculated larvae are dropped into the separate compartments. As soon as all compartments in a layer are occupied, additional soil is added to fill completely the compartments. A flat metal separator is placed on top of the filled layer, and a new cross section is then put in place in the box on top of the completed layer. The compartments in the new layer are partially filled with soil, injected larvae are placed in these compartments, and the whole process is repeated until all 5 layers in the box have been filled. The soil used to fill the boxes should contain, for each 100 pounds of soil, 1/2 pound of grass seed, which when sprouted will serve as food for the larvae during the incubation period.

The boxes are incubated at 86° F. for from 10 to 12 days. A high humidity should be maintained in the incubation chamber to prevent excessive drying out of the soil during incubation. The incubation boxes stacked in the incubating chamber are shown in figure 3.

(5) TREATMENT OF DISEASED LARVAE PRIOR TO MIXING WITH CARRIER

After incubation, the boxes are broken down, and the diseased grubs are screened out of the soil and dropped into a battery jar of ice water. The ice water inactivates the larvae, permitting thousands of them to be placed in the jar without danger of losses of spores due to nipping. The larvae are then washed in a colander to remove adhering soil particles, returned to the glass jar, packed with ice and held in a refrigerator at a temperature of approximately 32° to 35° F. until used, as shown in figure 4.

When sufficient numbers of diseased larvae have accumulated, the excess water is drained off the grubs, and the grubs are crushed by running them through a meat chopper. After all the larvae have been run through the chopper, the chopper is washed out with a small quantity of water to remove the adhering grub material. The resulting suspension, together with the wash water run through the chopper, is placed in a glass graduate and made up to even volume. Counts are made on this suspension, using a suitable dilution (1:2,000), and the density in spores per cc. is recorded.

(6) INCORPORATION OF THE SPORES WITH CARRIER

The standardized grub suspension is then added to the carrier (calcium carbonate, precipitated, U.S.P.) so that the mixture will contain a billion spores per gram of the dry material. The moist dust is then mixed thoroughly by running it through a mixing device, such as a blade cutter or trowel mixer. After thorough mixing has been accomplished, the moist dust is passed through a high-speed impeller-type blower, which shears the agglomerated particles.

Drying of the dust is accomplished by drawing heated air through the blower and exposing the finely divided particles to the warm air blast. This material when dry is the stable concentrated spore-dust preparation. The concentrate is then mixed with suitable quantities of dry carrier (talcum powder, marble flour, etc.) and stored until used. In the colonization work carried on by the Bureau, this final mixture contains 100 million spores per gram. Figure 5 is a photograph of the experimental plant-scale equipment used in preparing spore-dust material.

(7) FINAL CHECK ON THE SPORE CONTENT OF THE CONCENTRATED SPORE-DUST MIXTURE

The spore content of the dry spore-dust concentrate may be checked as follows: Ten grams of the powder are placed in a volumetric flask, 200-ml. capacity, approximately 100 ml. of distilled water added, the flask is shaken to wet the particles, and then 20 cc. of concentrated hydrochloric acid is added to the suspension. The flask is gently rotated with the stopper out until gas evolution ceases. The suspension is then made up to volume (200 ml.). The flask is stoppered and shaken vigorously. A loopful of this suspension is then used to fill the counting chamber. The count on the suspension should be 50 million spores per cc. The actual count in millions obtained is multiplied by 0.02 and the result recorded as the spore content in billions per gram of the concentrated dust mixture.

SUGGESTIONS AND PRECAUTIONS TO BE OBSERVED

1. The necessity of insuring the absence of any air entrapped either in the needle or syringe cannot be overemphasized. Air is easily detected by the operator and is evidenced by the formation of a droplet on the end of the needle when the larva is withdrawn.

2. In case of accidental puncture of the intestine of a grub during injection, the hypodermic needle should be sterilized immediately by swabbing with 0.5-percent sodium hypochlorite solution before proceeding with further inoculation. Otherwise subsequent larvae may be infected with septicemia-producing bacteria, contaminating the needle from the punctured gut.

3. Syringes should be flushed immediately after use with distilled water and then with alcohol to avoid a plugged needle or "freezing" of the plunger in the syringe.

4. Syringes should be returned to test tubes and the plugs replaced after use. Sterilization of glassware should be effected as soon as possible after cleaning.

5. Careful records should be made of numbers of larvae injected, spore and volume dosages, the number of larvae living at the end of the incubation period, the number diseased, the number not infected, and the yields in number of spores per grub. From such records it is easy to control the process fully and correct any serious faults in procedure as they occur. The saving this control work can effect will more than offset the additional time and effort expended.

APPENDIX

Method for preparing and adjusting density of spore suspensions for use in the inoculation procedure

In section 2, specific directions are given for the preparation of the inocula from stock culture slides. Reference is made on page 2 to the matter of determining the density of the spore suspension and the adjustment of the spore density to 300 million spores per cubic centimeter. It is believed that the procedure followed at the Japanese Beetle Laboratory in connection with these points may be of interest to other workers and, therefore, a detailed procedure is given herewith.

To prepare sufficient spore suspension to inoculate from 500 to 1,000 larvae, the following procedure will be found helpful. First prepare --

(A) Sample tube containing 1 cc. of sterile distilled water.

(B) Inoculum tube containing 2 cc. of sterile distilled water.

1. Wash spores from two culture slides into the inoculum tube (B) with an additional 1-cc. portion of sterile distilled water so that the total volume of the suspension is 3 cc.

2. Fill the capillary pipette with a loopful of this suspension to the 0.01-cc. mark. Discharge the capillary pipette into the sample tube (A). Shake the sample tube.

3. Withdraw a loopful of the suspension in the sample tube and fill the counting chamber. Place the counting chamber in the mechanical stage of the microscope and allow the chamber to stand 2 minutes to permit settling of the spores in the chamber.

4. Using a 4-mm. N. A. 0.65 objective, count the number of spores in 5 large squares of the Levy counting chamber (80 small squares).

5. The number of spores in 5 large squares multiplied by 5 equals the number of spores in millions per cc. of the suspension in tube (B).

6. This figure divided by 100 (pointing off two places) equals the volume to be made up to in order to have the desired concentration of 300 million spores per cubic centimeter of suspension. Subtract 3.00 to determine the volume of sterile distilled water which must be added.

For example: A spore suspension shows a count of 86 spores per 5 large squares. Multiplied by 5 this gives us the count as 430 million spores per cc. Or the suspension must be made up to 4.30 cc. to have the standard concentration of 300 million spores per cc. Substracting 3, this leaves 1.30 cc., the volume of sterile distilled water to be added to the suspension.

The above computations follow as below:

In the Levy counting chamber the dimensions of the small squares are 1/20 mm. in width and the depth of the chamber is 1/10 mm. Therefore, the volume of each small square is 1/4,000 cubic mm. or 1/4,000,000 cubic centimeter.

The number of spores in 5 large squares divided by 80 equals the mean number of spores in 1/4,000,000 of 1 cc. of the diluted suspension (1 cc. of the diluted suspension contains the spores from 0.01 cc. of the original spore suspension).

The number of spores per cc. of the actual spore suspension is, then,

$$\frac{\text{No. of spores in 5 large squares}}{80 \times 1/4{,}000{,}000 \times 0.01} =$$

$$\frac{\text{No. of spores in 5 large squares} \times 1{,}000{,}000}{20 \times 0.01} =$$

5 × No. spores in 5 large squares in millions per cc.

or, since there are exactly 3 cc., the total number of spores is 3 times the figure computed above.

Since we desire 300 million spores per cc., we divide the total number of spores determined by 300 to get the volume equivalent to this concentration. This will be equal to the number of spores counted multiplied by 5/100.

The general formula for counting with the Levy counting chamber is as follows:

$$\text{Spores per cc.} = \frac{\text{No. spores counted}}{\text{No. squares}} \times 4{,}000{,}000 \times \text{dilution}$$

The general formula for counting with chambers other than the Levy counting chamner will differ, depending on the chamber dimensions. For example, the Petroff-Hausser bacteria-counting chamber uses the same ruling as the Levy (improved Neubauer), but the depth of the chamber is 1/50 mm. instead of 1/10 mm. This

will change the factor in the general formula from 4,000,000 to 20,000,000, since the volume of a small square in the Petroff-Hausser chamber is 1/20,000,000 (1/20 × 1/20 × 1/50 × 1/1,000). The worker should acquaint himself with the chamber dimensions of the particular make of counting chamber which he proposes to use and determine the general formula to be used before making spore counts.

DETERMINATION OF PROPER SOIL MOISTURE TO BE USED IN PACKING INCUBATION BOXES

The use of soil at the proper moisture level is essential for satisfactory survival and spore production in injected larvae. Too low soil moisture will cause insufficient germination of the seed used for food and will prevent larvae from reaching maximum weight and hence will reduce the spore yield. Excessive soil moisture will increase larval mortality by interfering with the normal gas exchange in the soil. The absolute moisture value which is optimum for spore production and larval survival will depend on the soil type, sandy soils having a lower optimum value than heavier soils. It has been found that the optimum moisture value for a large number of soil types is that moisture which just prevents adherence of soil particles to the cuticula of the larvae. This is approximately equal to 60 percent of the "ball point" of the soil. Determination of the "ball point" of the soil is made as follows: Two 100-gram portions of air-dried soil are weighed into containers of about 250-ml. capacity, and water is added to them from a burette in 2-cc. increments, the soil being mixed thoroughly after each addition until it forms a plastic mass with just an excess of free moisture. When this point is reached, the soil ball formed will reform when the ball is broken up and the mass agitated by rotating the container. The amount of water added to bring the 100-gram sample to this state is recorded as the "ball-point" value of the soil, and 60 to 65 percent of this value is the moisture content which the soil should contain for use in the incubation boxes. These values are determined and expressed on the basis of grams (or cc.) of water per 100 grams of air-dry soil.

FOOD REQUIREMENT FOR SMALL THIRD-STAGE LARVAE

In section 4, describing the method of preparing larvae for incubation, ½ pound of grass seed per 100 pounds of soil is specified. This amount is adequate where full-grown third instars are used. If small or partly grown third instars are employed, this amount of seed is insufficient to insure maximum spore yields, and additional amounts of seed will be required. It would be well to use in such instances ½ pound of redtop grass seed and ½ pound of white Dutch clover seed per 100 pounds of soil. The more rapidly germinating clover seed would promote growth faster than redtop grass seed.

EQUIPMENT AND MATERIALS REQUIRED

The following equipment and materials will be required when 100,000 or more grubs are to be inoculated:

Microscopes.

2 binocular dissecting microscopes, with paired 10X eyepieces and 55mm. objectives, with microscope lamp for each.

1 compound microscope, equipped with mechanical stage, substage condenser Abbe type, 10X eyepieces, and 16, 4, and 1.8 mm. achromatic objectives, with microscope lamp.

1 steam-pressure sterilizer for sterilizing water blanks, syringes, pipettes, etc. (Sears-Roebuck 10-20 canner or similar will be suitable).

1 gas burner for sterilizer.

2 microinjectors, for injecting larvae (Dutky-Fest type). A limited number of these can be loaned by the Bureau to official agencies. These will have syringes, needles, and other necessary accessories.

Pipettes.

20 1-cc. pipettes with medium fine capillary tip.

1 0.01-cc. capillary pipette (Breed and Brew milk analysis type.)

6 each of 1-cc., 5-cc., 10-cc. pipettes, each graduated in 1/10 cc.

2 pipette cans.

1 Levy counting chamber and coverglass, double or single ruling (A. H. Thomas No 3300 or similar).

1 inoculating loop and loopholder, for filling counting chamber; loop should hold approximately 0.01 cc.

Test tubes.

50 16×150 mm. for water blanks, making up spore suspensions, etc.
10 20×150 mm. for syringe containers.

6 wire baskets for holding test tubes.

2 battery jars.

1 oil stone, small, fine grained, for repointing needles (Norton Pike Co. hard Arkansas oilstone, No. HB13, size 3, or similar).

Scales.

1 balance or table scale, 10-30 lb. capacity.

1 platform scale, 500-lb. capacity.

1 each graduated cylinder, 100-cc., 500-cc., 1-liter.

3 volumetric flasks or dilution bottles, 200-ml.

1 Bunsen burner.

60 incubation boxes, capacity 500 grubs each, size 10-3/16" x 10-3/16" x 5-3/4" deep (inside measurement), open top (sample can be furnished by the Bureau).

300 cross sections, capacity 100 compartments, each 1" x 1" x 1" (sample can be furnished).

360 dividers, 10" x 10", galvanized (sample can be furnished).

1 chopper, such as meat grinder.

Mixing equipment for collecting, mixing, and drying spore-dust mixture, similar to that shown in figure 5.

Drums, for storage of approximately 2 tons of spore-dust mixture.

Table space for injection work, not less than 4 feet of table space for each microinjector.

Seed, redtop, 25 lb. for grub food during incubation.

Soil, approximately 2-1/2 tons, to allow 25 lb. per incubating box.

Carrier (for spore), talc, 2 tons; calcium carbonate, precipitated, 450 lb.

Miscellaneous trays, screens, screening racks, and sieves for screening soil and sorting grubs. Refrigerator space for storing infected grubs.

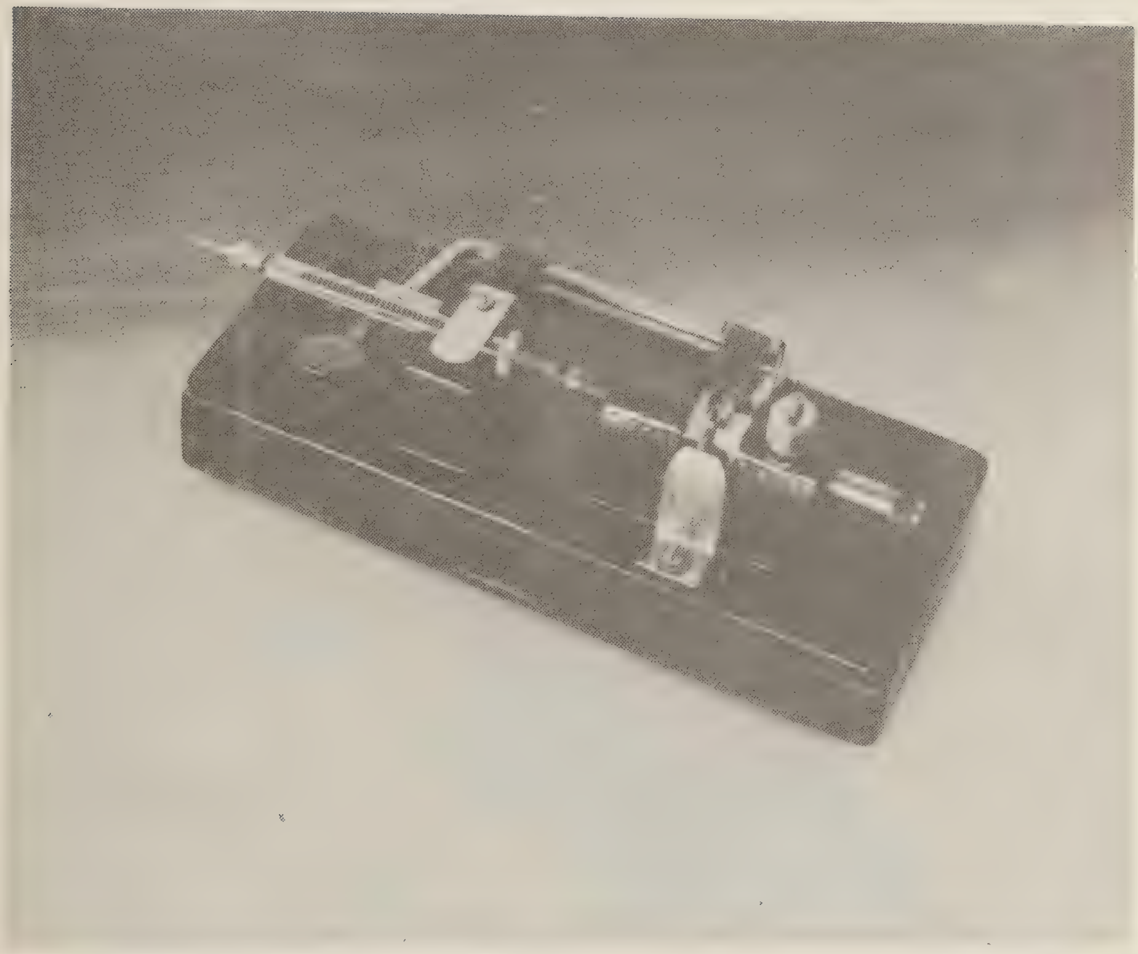

Figure 1.--Photograph of the Dutky-Fest microinjector with the syringe loaded and ready for use.

Figure 2.—Photograph illustrating the method of inoculating larvae with type A milky disease spores.

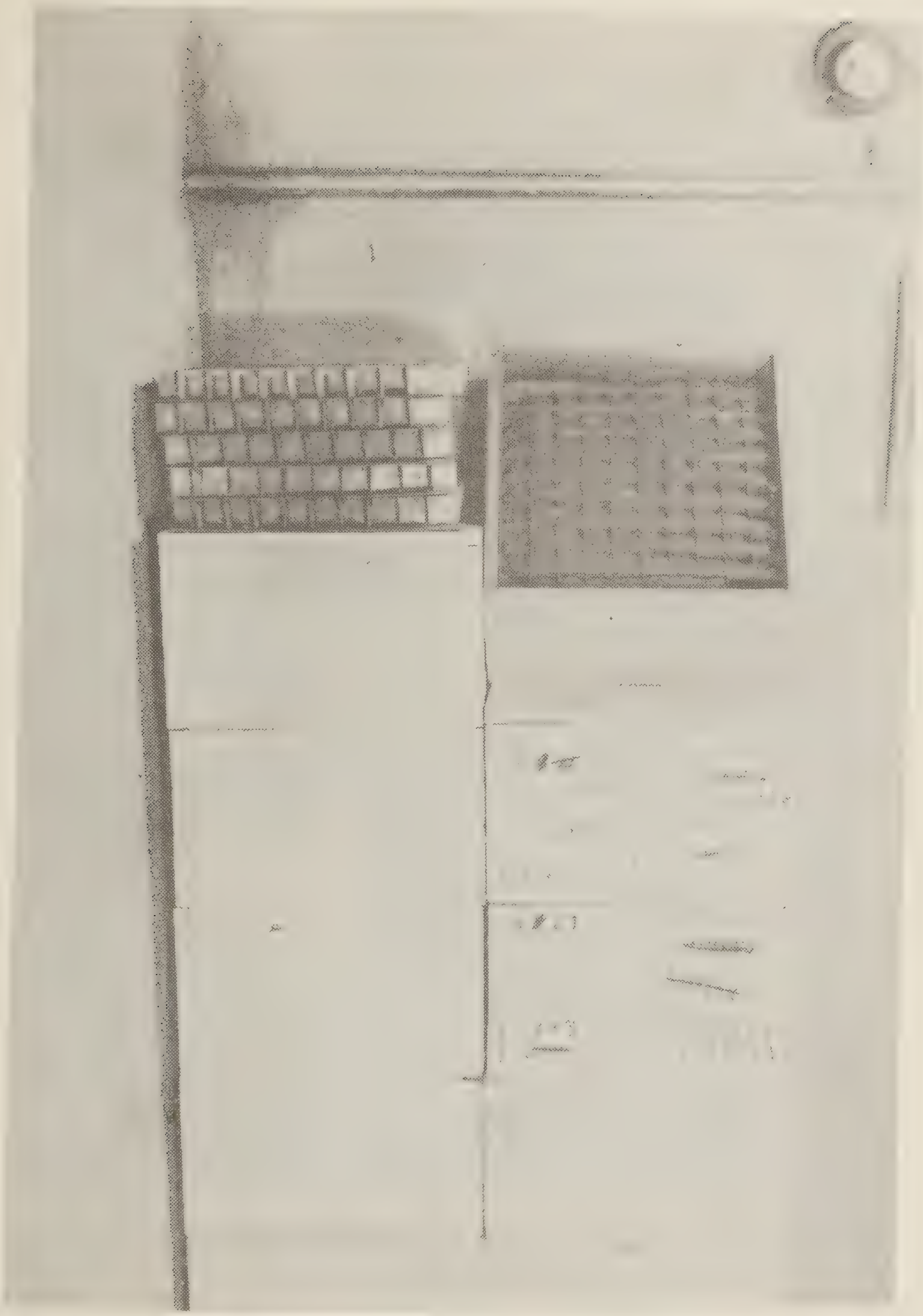

Figure 3.--Photograph of incubation boxes stacked in the incubating chamber. Note the alternately stacked cross sections and metal flats which divide the incubation boxes into 500 one-inch cubes.

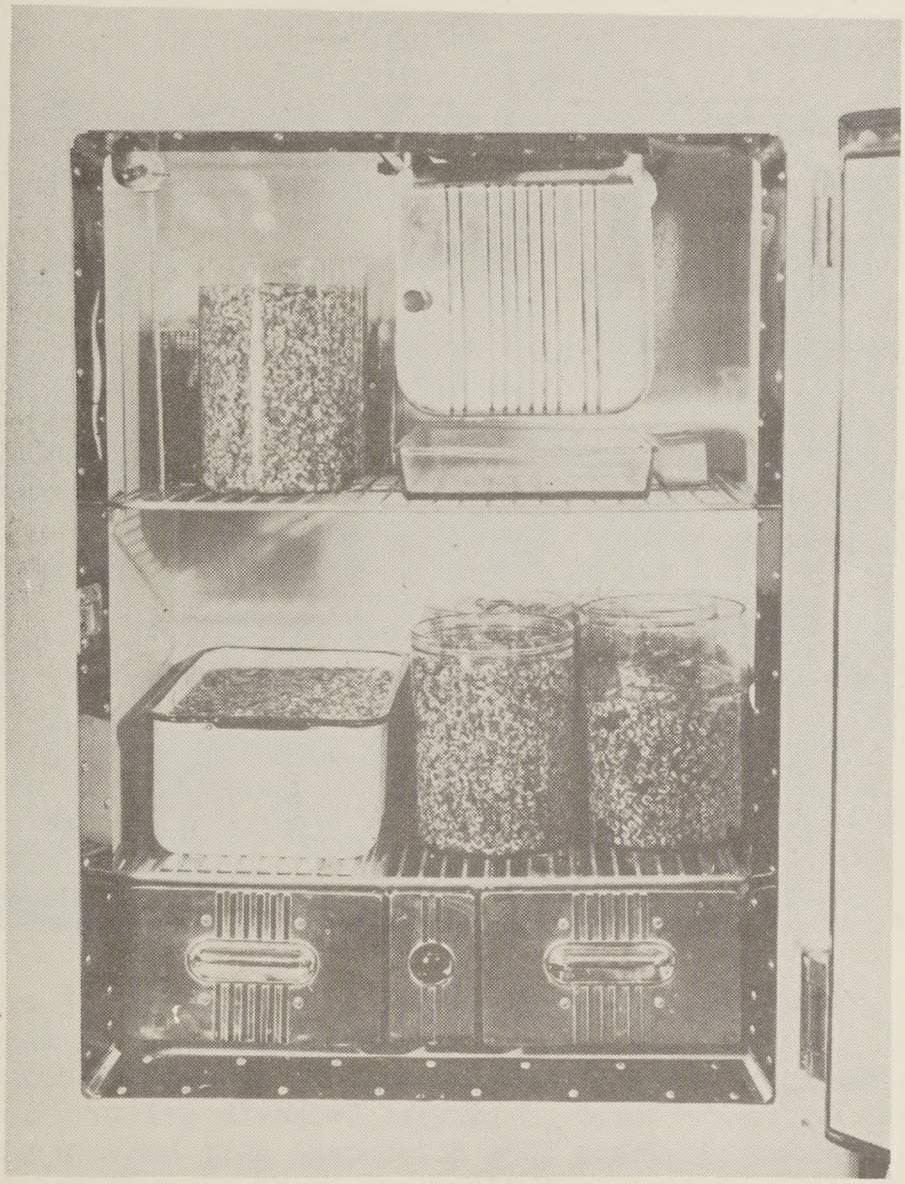

Figure 4.--Photograph of ice-packed jars of diseased larvae stored in the refrigerator. About 100,000 larvae may be stored in the 8-cubic-foot refrigerator shown.

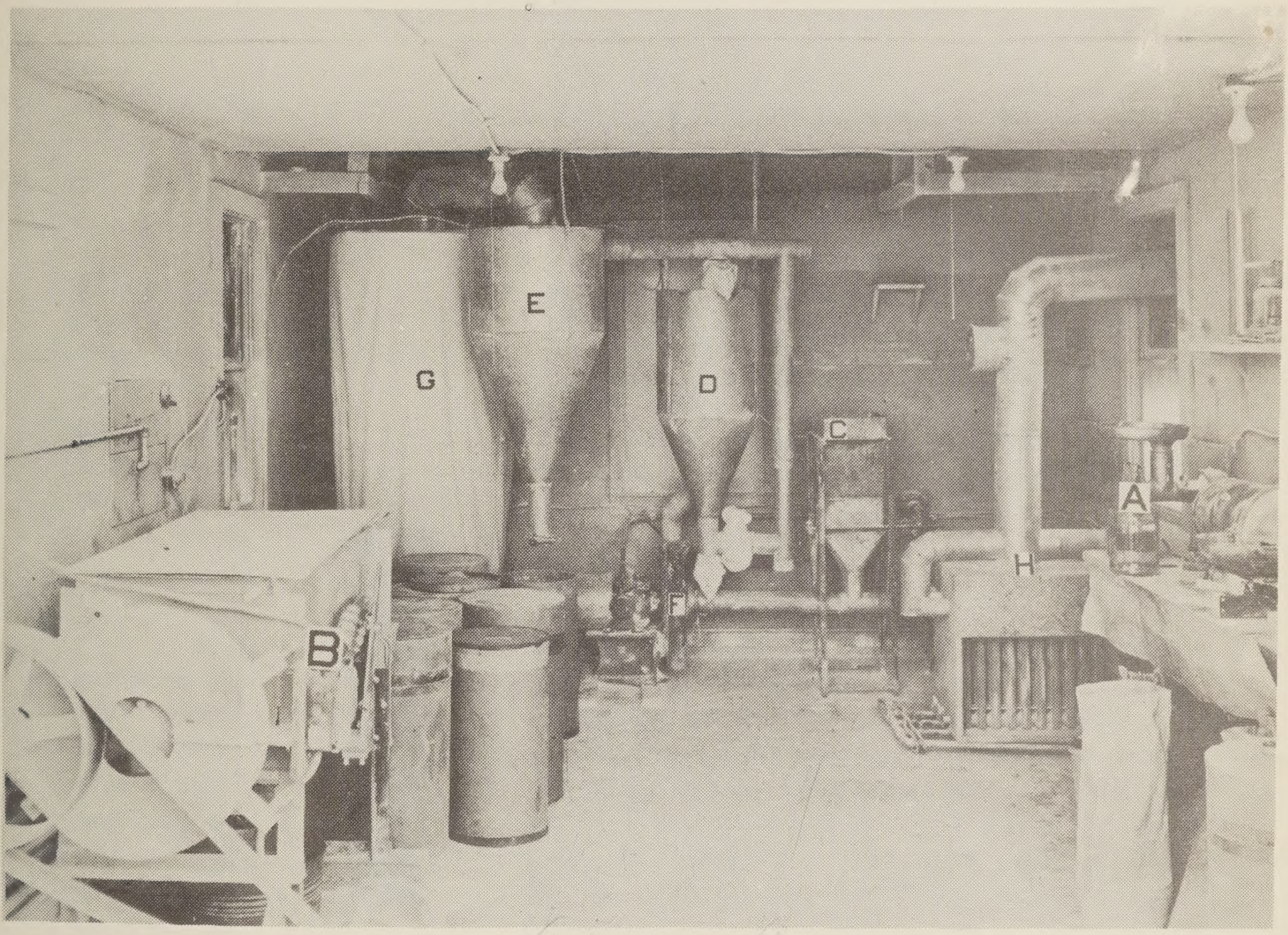

Figure 5.--Photograph of the experimental plant-scale equipment used in processing diseased grubs. The letters refer to the various elements of equipment as follows: A, Meat grinder for crushing diseased larvae; B, batch mixer for incorporating crushed grub suspension with precipitated chalk; C, hopper through which the preliminary mix is fed; D, classifying chamber (note the muslin-covered blast gates for controlling direction and quantity of air flow); E, cyclone dust collector; F, high-speed paddle-wheel impeller pressure fan; G, bag collector; H, gas-fired furnace for heating air blast for flash drying moist dust.